Denatured Collagen Modulates the Phenotype of Normal and Wounded Human Skin Equivalents

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Epithelial–mesenchymal interactions are known to play an important role in modulating homeostasis and repair. However, it remains unclear how the composition of the extracellular matrix may regulate the ability of dermal fibroblasts to engage in such cross talk. To address this, we studied how fibroblast phenotype was linked to the behavior of normal and wounded human skin equivalents (HSE) by comparing human dermal fibroblasts (HDF) incorporated into the three-dimensional tissues to those extensively cultivated in two-dimensional (2D) monolayer culture on denatured collagen (DC) matrix, native collagen, or tissue culture plastic before incorporation into HSEs. We first established that prolonged passage and growth of HDF on DC increased their migratory potential in a 2D monolayer culture. When HDF variants were grown in HSEs, we found that extended passage on DC and incorporation of DC directly into the collagen gel enhanced proliferation of both HDF and basal keratinocytes in HSEs. By adapting HSEs to study wound reepithelialization, we found that the extended passage of HDF on DC accelerated the rate of wound healing by 38%. Thus, extensive *ex vivo* expansion on DC was able to modify the phenotype of skin fibroblasts by augmenting their reparative properties in skin-like HSEs.

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INTRODUCTION

Fibroblasts are known to play an active role in regulating the development, maturation, and repair of human skin by synthesizing extracellular matrix (ECM) proteins (Clark *et al.*, 2007) and through the production of paracrine-acting factors (Werner *et al.*, 2007). However, how direct interactions between the ECM microenvironment and dermal fibroblasts modulate these fibroblast functions remains to be elucidated. It is therefore of interest to further understand how fibroblasts respond to their surrounding ECM to maintain skin homeostasis and during reestablishment of epithelial integrity after wounding. Previous studies designed to assess how the ECM microenvironment controls fibroblast phenotype have been performed in two-dimensional (2D) monolayer culture. It has been shown that prolonged *ex vivo* expansion on denatured type I collagen can modulate the cellular properties of human mesenchymal stem cells (Mauney *et al.*, 2004, 2005). However, the impact of direct interactions between fibroblasts and ECM proteins need to be studied in three-dimensional (3D) tissues that more closely mimic skin *in vivo*, in which epithelial–mesenchymal interactions can be recapitulated and monitored.

The development of tissue-engineered models that mimic human skin (human skin equivalents, HSEs) has provided biologically meaningful experimental systems to study epidermal biology and epithelial–mesenchymal cross talk in an *in vivo*-like tissue context (Andriani *et al.*, 2003; Abraham *et al.*, 2004, 2007; Margulis *et al.*, 2005). These 3D tissues allow direct determination of key response parameters of wounded epithelium, including cell proliferation, migration, differentiation, growth factor response, and protease expression (Singer and Clark, 1999) and provide a valuable model to study cell interactions in complex tissues.

In this study, we have used intact and wounded HSEs to study the capacity of fibroblasts passaged extensively or exposed to ECM composed of either normal collagen (NC) or denatured collagen (DC) to modulate the phenotype of these tissues. We found that only after extended passage on DC, dermal fibroblasts significantly modified their morphology and migration rate in 2D cultures. When these cells were incorporated into HSEs, they were able to modify the properties of the adjacent surface epithelium by increasing the proliferation of basal keratinocytes and significantly shortened the time needed for wounded HSEs to undergo complete reepithelialization. These results demonstrated that cell modifications mediated by direct interactions with their

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Abbreviations: 2D, two-dimensional; 3D, three dimensional; DC, denatured collagen; ECM, extracellular matrix; HDF, human dermal fibroblast; HDF-EP, early-passage HDF; HSE, human skin equivalent; NC, normal collagen; TCP, tissue culture plastic

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immediate ECM microenvironment hold tremendous potential for previously unidentified therapeutic applications and for understanding mechanisms through which cross talk between fibroblasts and keratinocytes may accelerate wound repair and reestablish tissue homeostasis applications.

RESULTS

Growth on denatured collagen matrix augmented migration of primary human dermal fibroblasts in 2D culture

To study the effect of ECM modulation of fibroblast phenotype in 3D HSEs, we first prepared these cells for incorporation into tissues through extended culture on various ECM substrates in 2D cultures. As has been recently reported that the nature of the type I collagen substrate directs fibroblast function in 2D culture (Volloch and Kaplan, 2002; Mauney *et al.*, 2004, 2005, 2006), we compared the effect of different collagen on the morphology and migration of primary human dermal fibroblasts (HDF). Early-passage HDFs (HDF-EP), initially expanded for 24 population doublings, were expanded for 12 additional passages (total of ~64 population doublings) on the following substrates: 1—non-coated tissue culture plastic (HDF-TCP), 2—a film of 0.5 mg ml^{-1} native collagen (HDF-NC), and 3—a film of 0.5 mg ml^{-1} denatured collagen (HDF-DC). After these 12 passages, HDF-TCP and HDF-NC cells underwent well-defined morphological changes. Initially, slender HDF-EP cells (Figure 1a) increased in size with passaging as seen by the flattened morphology of cells passaged on tissue culture plastic (Figure 1d) and NC (Figure 1b). These cells spread upon attachment to occupy an area twice the surface area observed for HDF-EP cells (Figure 1e). In contrast, HDF-DC cells showed a morphology that was similar to the HDF-EP cells after 12 passages on a matrix of DC (Figure 1a and c). Thus, morphologic analysis revealed that prolonged culture of HDF cells on DC matrix allowed cells to retain their initial phenotype when compared to changes that occurred upon extended passage on non-coated and NC-coated dishes.

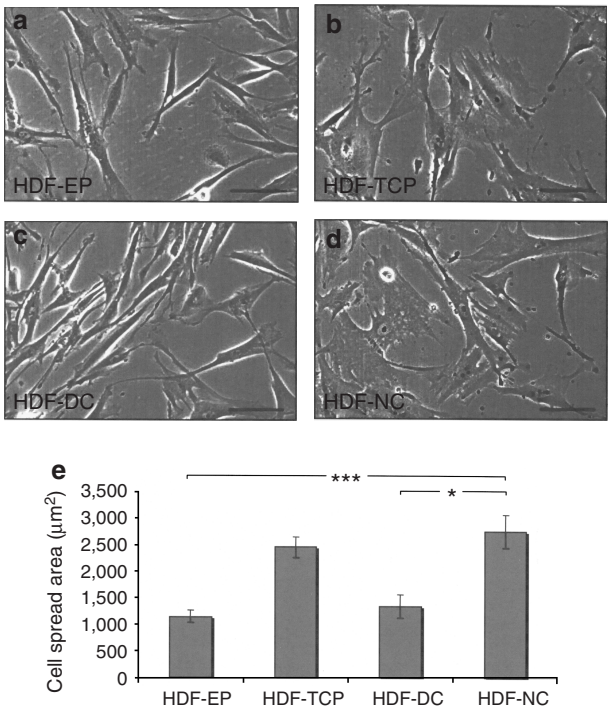

Figure 1. Prolonged growth of HDF cells on a denatured collagen matrix decreased passage-related morphological changes. (**a**) HDF-EP cells, passage 8, were maintained for 12 additional passages on the following substrates: (**b**) tissue culture dishes (HDF-TCP), (**c**) film of 0.5 mg ml^{-1} denatured collagen (HDF-DC), and (**d**) film of 0.5 mg ml^{-1} native collagen (HDF-NC). (**e**) Cell area represents the mean and SD of $N=20$ independent determinants. Bar = 100 μm. ***$P<0.001$, *$P<0.01$ (*t*-test).

As it is known that extended passage may reduce both the migration and proliferation of fibroblasts *in vitro* or *in vivo* (Muggleton-Harris *et al.*, 1982; Lombard and Masse, 1987), we next studied whether these morphologic differences were linked to an increased proliferative rate and/or motility of HDF-DC cells. We performed scrape wound assays, in which confluent cell monolayers were mechanically disrupted to create an area devoid of cells. When the rate of cell repopulation was measured, both HDF-EP cells (Figure 2a) and HDF-DC cells (Figure 2b) were found to repopulate the wound gap 12 hours after wounding. In contrast, HDF-NC cells did not repopulate the wound gap, as only a few of these cells migrated into the wound after 12 hours (Figure 2c). As summarized in Figure 2g, quantitative analysis of scrape wounds revealed a 29% increase in the rate of cell migration of HDF-DC cells in comparison to HDF-NC cells (HDF-EP = 100%, HDF-DC = 82%, HDF-NC = 53%). We also measured the fibroblast proliferation rates inside the wound gap, 12 hours after wounding using a pulse of BrdU to determine the contribution of cell growth to repopulation of the wound gap. We found that HDF-EP cells (6.4 ± 0.6%) and HDF-DC cells (6.8 ± 0.8%) showed a similar increase in BrdU labeling when compared to HDF-NC cells (2.4 ± 0.8%, $P>0.0001$). These results are consistent with the notion that extended passage of HDF on a DC matrix could alter migratory potential, this may be explained by both augmented cell motility as well as by increased cell proliferation.

Exposure to DC enhances the proliferation of both human fibroblasts and basal keratinocytes in dermal equivalents

To further establish the significance of findings in 2D monolayer culture, the phenotype of HDF-DC and HDF-NC in a more biologically meaningful environment was assessed by incorporating these cells into 3D collagen gels that mimic the dermal compartment of human skin (dermal equivalents). To directly establish how NC or DC could modulate the phenotype of HDFs not previously passaged (HDF-EP), we fabricated dermal equivalents with these cells in either 10% DC or 25% DC that was mixed with NC and compared them with dermal equivalents fabricated only with NC. After 1 week, dermal equivalents were pulsed with BrdU and the proliferation of fibroblasts was determined by immunohistochemistry. We observed a direct correlation between HDF-EP proliferation and DC concentration. As seen in Figure 3, the proliferation index of HDF-EP cells increased from 14.8 ± 4.5% for an NC microenvironment to 30.8 ± 5% when cells were grown in a dermal equivalent consisting of 10% DC. The proliferative index increased to 45 ± 7% when

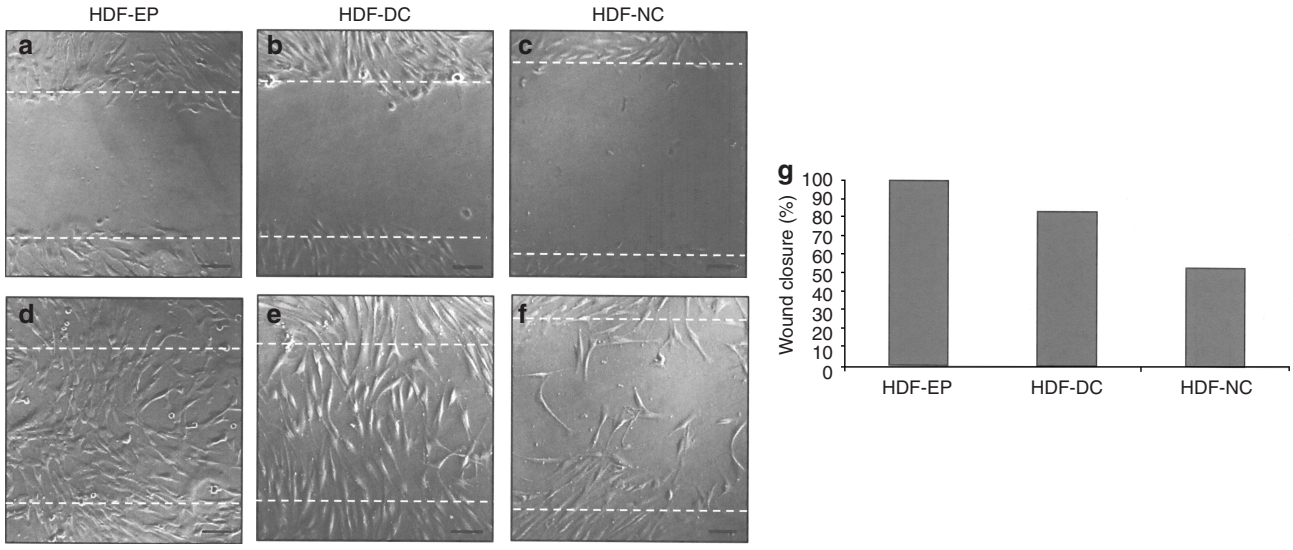

Figure 2. Prolonged growth of HDF cells on a denatured collagen matrix decreases the rate of passage-related changes in cell motility. An initial scratch wound was carried out on confluent cultures of (**a**) HDF-EP cells and on cultures of (**b**) aged HDF-DC cells or (**c**) HDF-NC cell. (**d–f**) Repopulation of the wound surface by (**d**) HDF-EP cells, and (**e**) aged HDF-DC cells or (**f**) HDF-NC cells after 12 hours. (**g**) Cells that migrated into the wound gap were calculated for each condition and differences were expressed as a percentage of wound closure of the early passaged HDF cell culture (100%). Each point represents the mean and SD of triplicate determinations in two separate experiments. Bar = 100 μm.

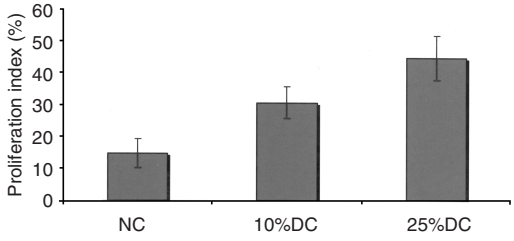

Figure 3. Denatured collagen enhances the proliferation of HDF cells incorporated into dermal equivalents. The proliferation rate of dermal equivalents, incorporating either HDF-EP and native collagen (NC), 10% denatured collagen (10% DC) or 25% denatured collagen (25% DC) was measured as the percentage of total cells that were BrdU-positive. Two independent experiments with a minimum of three observations for each condition were analyzed.

DC was 25% of total collagen in the gel. This demonstrated that DC could modulate the growth of HDF in dermal equivalents even without extended passage on this substrate.

We next determined whether this response was also seen in 3D tissues using HDF that underwent extended 2D passage on either DC or NC before incorporation into dermal equivalents. Tissues were constructed by incorporating HDF-EP, HDF-DC, or HDF-NC cells into collagen gels and culturing them for 7 days (Figure 4a–c). When dermal equivalents were pulsed with BrdU at this time and proliferation analysis was performed, HDF-DC revealed a 5.8-fold increase in proliferation when compared to tissues grown with HDF-NC cells (HDF-NC = 0.76 ± 0.7%) or HDF-EP (HDF-EP = 1.9 ± 1.3%, HDF-DC = 5.2 ± 1.8%), further suggesting that exposure to DC in either 2D culture or 3D tissues maintained HDF proliferation.

Our previous studies have shown that the presence of dermal fibroblasts supported the proliferation of basal keratinocytes in HSEs (Andriani *et al.*, 2003). To assess if passage on DC could alter the ability of fibroblasts to modulate keratinocyte growth, the proliferation and morphology of keratinocytes was analyzed after constructing HSEs with the three types of HDFs. All three fibroblast subtypes generated HSEs that were well stratified, fully differentiated with evidence of normal epithelial tissue architecture (Figure 5a–c). When the proliferation rate of basal keratinocytes was determined, it was found that keratinocytes grown in the presence of HDF-NC showed the lowest proliferative index (0.5 ± 0.5%), whereas tissues grown with HDF-EP showed proliferation that was ninefold higher (4.5 ± 0.4%). Significantly, the PI for basal keratinocytes grown in the presence of HDF-DC was nearly 20-fold higher than that shown for HDF-NC (9.5 ± 2.8%). This elevation in proliferation is reflected in the morphology of basal cells (Figure 5). Tissues grown with HDF-DC demonstrated basilar hyperplasia, in which basal keratinocytes appear denser and more columnar when compared to tissues constructed with HDF-NC and HDF-EP. These results show that cross talk between fibroblasts and keratinocytes in HSEs was associated with increased proliferation of basal keratinocytes. As only HDFs grown on DC were able to significantly increase the proliferation rate of basal keratinocytes, it appears that HDF keratinocyte may activate and sustain tissue growth.

HDF cells passaged on DC matrix accelerate the rate of wound healing in skin equivalent cultures

Proliferation of activated keratinocytes distal to the wound margin is known to be the driving force that enables rapid wound reepithelialization (Garlick and Taichman, 1994b; Garlick *et al.*, 1996). In light of this, we used our previously

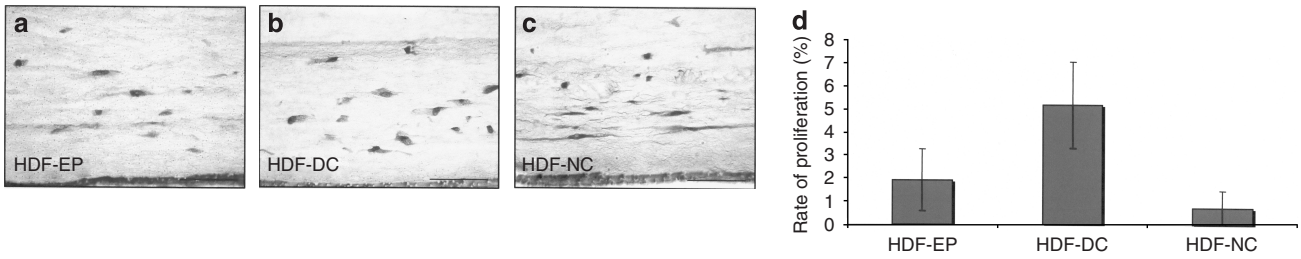

Figure 4. Morphologic appearance and proliferation rate of the HDF cells cultivated on collagen gels. (**a–c**) Collagen gels populated by (**a**) HDF-EP cells, (**b**) aged HDF-DC cells, (**c**) or HDF-NC cells. (**d**) The proliferation rate was measured by BrdU labeling and expressed as a percentage of total cells. Two independent experiments with a minimum of three observations for each condition were analyzed. Bar = 50 μm.

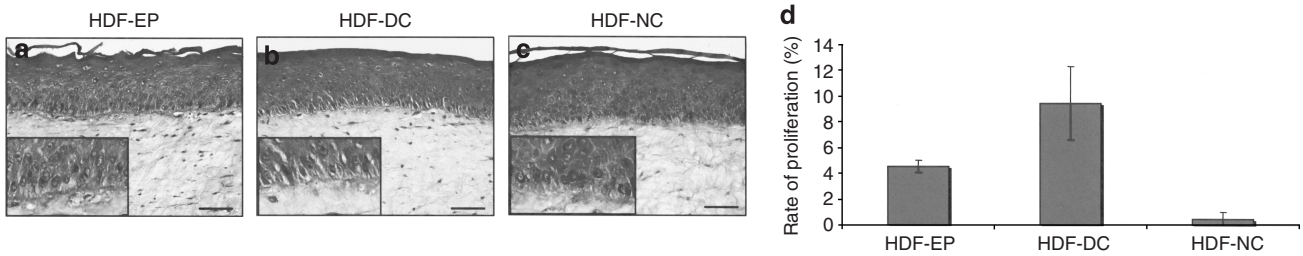

Figure 5. Proliferation rate of the basal keratinocytes of skin equivalents is influenced by HDF cells populating in the collagen gels. To generate skin equivalents, normal human keratinocytes (NHK) cells were grown on the top of collagen gels populated by (**a**) HDF-EP cells or (**b**) aged HDF-DC cells, and (**c**) HDF-NC cells. An enlargement of each picture shows the morphology of basal keratinocytes. The rate of proliferation of basal keratinocytes was measured by performing BrdU labeling after 14 days of culture. (**d**) Differences were expressed as a percentage of total basal keratinocytes that were BrdU-positive. Two independent experiments with a minimum of three observations for each condition were analyzed. Bar = 50 μm.

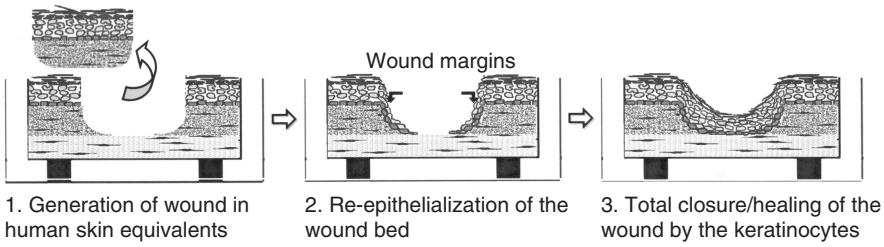

Figure 6. Schematic diagram of 3D wound model of skin equivalent cultures. (1) A wound was created by excising a central portion of human skin equivalent cultures. (2) Keratinocytes from the edge of the wound move across the wound bed to repopulate it. (3) Reepithelialization leads to reestablishment of epithelial integrity as keratinocytes reconstitute a fully stratified epithelium.

developed 3D HSE model of cutaneous wound repair (Garlick and Taichman, 1994b) to determine whether HDF-DC could also modulate the rate of reepithelialization of wounded HSEs. To test this, HSEs containing HDF-EP, HDF-TCP, HDF-DC, or HDF-NC cells were wounded as described in Figure 6. The rate of wound reepithelialization was assessed by comparing the degree of reepithelialization from the wound edge 24, 48, and 72 hours after wounding. After 24 hours, epithelial tongues were seen at the edge of the wound margin, indicating reepithelialization was initiated at this time (data not shown). Forty-eight hours after wounding, the wound bed was partially covered by a thin layer of keratinocytes in the central area of the wound and a more stratified epithelium toward the wound margins (Figure 7a–d). By 72 hours after wounding, the wound bed was completely covered by keratinocytes and epithelial integrity was restored (data not shown). To determine whether the rate of wound healing differed in the presence of different HDF subtypes, the distance separating the two epithelial tongues at the edge was measured and normalized to the location of the initial wound margins (Figure 7e). HDF-DC showed a rate of wound closure similar to tissues in which HDF-EP cells were incorporated (HDF-EP = 95.75 ± 3.3% and HDF-DC = 90.50 ± 10.1%). In contrast, HDF-TCP and HDF-NC showed a significantly slower rate of reepithelialization (HDF-TCP = 65.50 ± 5.2% and HDF-NC = 66.00 ± 10.1%). This indicates that incorporation of HDF cells passaged on DC matrix led to an accelerated rate of wound healing when compared with cells that were passaged on NC or tissue culture plastic. This demonstrates that extended passage on DC allowed HDF to retain their capacity to optimally direct reepithelialization, a feature that was lost when HDF cells were passaged on a native type I collagen substrate.

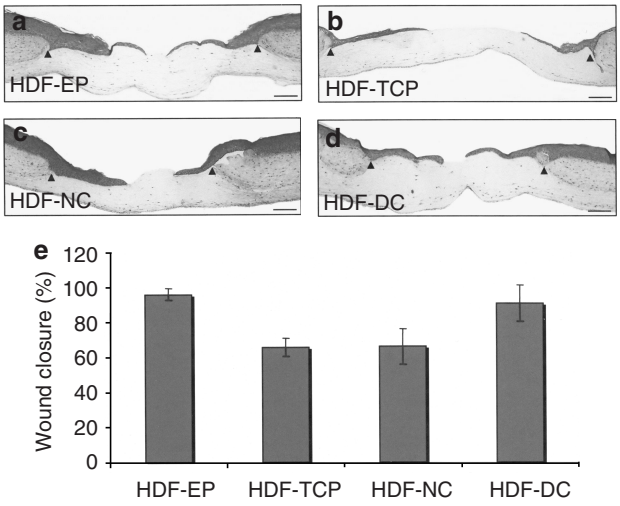

Figure 7. HDF-DC cells accelerate the rate of wound reepithelialization of wounded skin-equivalents. (**a**–**d**) The morphology of skin equivalent cultures fabricated using (**a**) HDF-EP cells, (**b**) HDF-TCP, (**c**) HDF-NC, or (**d**) HDF-DC cells 48 hours after wounding. Arrows demarcate the initial wound edges. (**e**) The degree of reepithelialization was determined by comparing the cultures immediately after wounding with those seen 48 hours later and expressed graphically as the percentage of wound closure. Two independent experiments with a minimum of three observations for each condition were analyzed. Bar = 100 μm.

DISCUSSION

Epithelial–mesenchymal interactions are known to regulate the development, homeostasis, and repair of human skin (Clark *et al.*, 2007). Most studies on cellular interactions mediating these events have focused on growth factor-mediated cross talk between keratinocytes and fibroblasts (Werner *et al.*, 2007). However, it still remains unclear how the ECM surrounding these fibroblasts can impact their ability to direct such cross talk. This is in part due to these diverse, mature, and poorly characterized phenotypes of dermal fibroblasts (Azzarone and Macieira-Coelho, 1982; Wright *et al.*, 1991). For example, fibroblasts in the more superficial layers of the dermis have shown elevated expression of types I and III collagen when compared to cells in the deeper dermis (Ali-Bahar *et al.*, 2004). It is thought that fibroblast phenotype is sensitive to such differences in dermal ECM composition (Fries *et al.*, 1994; Eckes *et al.*, 2006). In addition, dynamic changes in ECM composition that occur after wounding are known to regulate wound fibroblast phenotype (Clark, 1993). In this light, we have investigated how the composition of the ECM modulates both fibroblast phenotype and their capacity to engage in cross talk with either intact or wounded cutaneous epithelium.

Complex 3D tissues, such as HSEs, provide a powerful tool to identify mechanisms through which epithelial–mesenchymal interactions modulate keratinocyte growth and differentiation (Maas-Szabowski *et al.*, 2001). It is known that biologically meaningful signaling pathways, mediated by linking of adhesion and growth, function optimally when cells are spatially organized in 3D tissues, but are uncoupled and lost in 2D culture systems (Bissell and Radisky, 2001). Therefore, in this study, it was essential to fabricate 3D tissues that display the architectural features seen in *in vivo* tissues to further understand the effect of the ECM microenvironment on HDF in their appropriate tissue context.

HSEs have been used previously to demonstrate that the phenotype of regulated epithelium is by the reciprocal modulation of growth factor expression in fibroblasts and keratinocytes. It was shown that fibroblast-mediated, paracrine control of keratinocyte proliferation was linked to keratinocyte growth factor and GM-CSF production by fibroblasts (Maas-Szabowski *et al.*, 2001) that directly augmented keratinocyte proliferation in HSEs. However, HSEs have not been used to determine whether alteration of fibroblast–ECM interactions can also induce such phenotypic changes in the surface epithelium. To address this, we have adapted HSEs to determine that phenotypic modulation of HDFs after extended passage on a DC substrate could direct outcomes of intact and wounded skin-like HSEs. We found that the tissue phenotype of HSEs was able to sustain a more proliferative tissue phenotype when DC-passaged cells were incorporated into HSEs when compared to NC-passaged cells. The fact that DC-passaged cells evoked tissue outcomes that were similar to fibroblasts, which were not passaged (HDF-EP), suggests that interactions with DC preserved cellular functions that can be lost upon cell senescence. In light of the tremendous variability seen in fibroblasts isolated from dermis and grown in 2D monolayer culture (Wright *et al.*, 1991), it is possible that a subset of fibroblasts was selected by exposure to DC. Such clonal heterogeneity in fibroblast phenotype is well documented and may be related to a dynamic balance that favors selection of a particular fibroblast subpopulation within a specific ECM environment that will then dictate its phenotype and cross talk with the surface epithelium. This suggests that the relative proportion and stromal distribution of distinct fibroblast subtypes may be directed by specific fibroblast–ECM interactions and may play a critical role in the control of stromal and epithelial function in healthy and wounded skin. However, the mechanisms through which HDF functions are controlled by ECM remain to be explored. Previous studies have shown that prolonged passage of mesenchymal cells on DC could modulate cell phenotypes in 2D monolayer cultures (Volloch and Kaplan, 2002). However, to our knowledge, the use of HSEs to show that the phenotype of both normal and wounded tissues can be modulated by direct contact between HDF and specific ECM proteins has not been reported previously. By extending previous observations made in these rudimentary 2D culture systems to those gleaned from biologically relevant 3D tissue context, future applications can take advantage of such cellular plasticity to construct engineered human tissues with optimal function that will enhance human wound repair.

Previous studies performed in 2D culture have demonstrated that heat denaturation of type I collagen resulted in the dissociation of its triple helical structure (Har-el and Tanzer, 1993), leading to the exposure of internal RGD (Arg-Gly-Asp) cell adhesion sequences that are inaccessible in the native type I collagen conformation (Yamamoto *et al.*, 1995). Several studies of cell adhesion to type I collagen have

shown that cells primarily adhere to the native structure through RGD-independent integrin interactions, whereas RGD-dependent binding of cells can be observed following the denaturation process (Aumailley *et al.*, 1989; Vandenberg *et al.*, 1991; Gullberg *et al.*, 1992; Pfaff *et al.*, 1993). Taken together with our previously published (Volloch and Kaplan, 2002; Mauney *et al.*, 2004, 2005) and current results, these observations suggest the possibility that RGD-dependent integrin interactions specific for adhesion to the DC matrix may play a significant role in cellular signaling that are required for the retention of cellular functions normally lost upon extensive cell passage. In addition, it is possible that DC-mediated adhesion to type I collagen regulates matrix metalloproteases (MMP) function (Heino, 2000), releasing ECM fragments and exposing cryptic epitopes (Mauney *et al.*, 2006) that can modulate cell growth and maintain other cellular functions that are normally lost upon extensive cell passage. In addition, our findings are interesting in light of previous studies that have shown that cell adhesion to DC is dependent on fibronectin. It is known that newly synthesized fibronectin or fibronectin present in serum-containing media promotes adhesion of both myogenic (Hauschka and White, 1972) and Hamster kidney cells (Grinnell and Minter, 1978) on a gelatinized substrate through binding to the α1(I) chain of collagen. The relevance of these findings to our studies is evidenced by the demonstration that the fibronectin-dependant attachment of HeLa cells only occurred on DC, whereas attachment to NC was fibronectin-independent (Schor and Court, 1979). Thus, it is possible that changes in fibroblast phenotype characterized in our study may also be mediated through interactions with other ECM proteins, such as fibronectin.

Although a good deal is understood about keratinocyte phenotype during skin development and wound repair, less is clear about how the phenotype of fibroblasts are altered during these events after wounding (Clark *et al.*, 2007; Werner *et al.*, 2007). We have adapted HSEs to study reepithelialization and wound response in a tissue that mimics the chronology of events that occur after wounding of human skin (Garlick and Taichman, 1994a, b; Garlick *et al.*, 1996). It is now well established that the ECM microenvironment plays a critical role in the maintenance of normal tissue phenotype, as disruption of this ECM control can precipitate disease initiation and progression. However, it is less clear how the ECM microenvironment modulates cell phenotype to direct the phenotypic properties of keratinocytes. Our findings show that dermal fibroblasts manifest plasticity that allows their phenotype to be controlled by ECMs of varying composition. This supports recent findings that tissue stiffness, as a function of ECM composition, can direct mesenchymal stem cells to specific tissue outcomes (Eckes *et al.*, 2006; Engler *et al.*, 2006). The significance of this cellular plasticity for tissue engineering applications is profound, as it may provide new methodologies for expansion of progenitor populations that can amplify specific cellular phenotypes that may impact long-term tissue outcomes. Furthermore, the finding that the DC microenvironment maintains features of early-passage fibroblasts will allow us to further understand how ECM–cellular interactions may limit cellular and tissue aging processes. Such insights have implications for modulating age-related changes in aging at the cellular and tissue levels.

There are numerous biomedical applications that stem from our observations. Extended passage of cells on DC may improve autologous cell transplantation by optimizing the isolation, selection, cultivation, and expansion of specific subsets of dermal fibroblasts that retain advantageous cellular and tissue properties. Another potential application may be the direct application of DC gels into wounds to modulate epithelial–mesenchymal cross talk to increase the rate of healing. In the past, collagen materials in the form of gels, scaffolds, and films have been applied, either alone or in combination with other agents, to advance soft tissue augmentation and wound repair (Clark *et al.*, 2007). On the basis of our results, the addition of DC in such devices might increase the quality of tissue repair by triggering similar cellular responses to those observed in our studies. Direct application of such cells prepared by their selective interaction with specified ECM components may be important for future therapies relevant to patients at elevated risk for development of chronic wounds.

In summary, we have demonstrated that interactions between dermal fibroblasts and DC matrices lead to the retention of phenotypic properties that sustain cell proliferation and demonstrate a higher rate of wound healing that is comparable to non-passaged cells when cultivated in 3D human skin-like tissues. Our findings show that regulated ECM–fibroblast interactions provide a promising interface for future therapeutic applications.

MATERIALS AND METHODS

Cells

HDFs were derived from newborn foreskins as described previously (Rovee and Maibach, 2004). Cells were seeded at a density 5×10^4 cells ml^{-1} and cultures were sequentially passaged when cell density reached confluence. Passage 8 HDFs (HDF-EP) were maintained for 12 additional passages on tissue culture plastic (HDF-TCP), DC films (HDF-DC), or NC films (HDF-NC). Normal human keratinocytes were isolated from newborn foreskin and maintained in keratinocyte medium described by Wu *et al.* (1982) and grown on irradiated 3T3 fibroblasts.

Preparation of DC films

Type I collagen from rat tail (cat no. 1179179; Roche, Manheim, Germany) was dissolved at 5 mg ml^{-1} in 0.1% acetic acid and denatured by incubation at 50 °C for 12 hours resulting in complete collagen denaturation that was previously confirmed using circular dichroism measurements to demonstrate a thermal transition at 45 °C (Volloch and Kaplan, 2002). In addition, gel analysis of collagen denatured at 50 °C for various time periods showed that after 12 hours of treatment, the bulk of collagen remained intact in terms of molecular weight (Volloch and Kaplan, 2002). To prepare films for cell passage, 1.5 ml of collagen solution (0.5 mg ml^{-1}) was added to 35 mm tissue culture plates (Corning, Lowell, MA) and dried under vacuum. As a control, native collagen films (0.5 mg ml^{-1}) were prepared in the same way.

Cell morphology and spread area quantitation

Digital images of cells were captured using a Zeiss Axiovert 40C microscope, equipped with PixeLINK PL-A662 camera. Cell areas were determined automatically after tracing around the perimeter of individual cells using SPOT Advanced Image Analysis software. For each sample, 20 randomly selected cells were analyzed.

Scratch wound healing assay

Passaged HDF-DC or HDF-NC cells were seeded on DC films or native collagen films. As a control, HDF-EPs (passage 8) were seeded on untreated tissue culture plate. After reaching confluence, cells were wounded with a sterile 0.1- to 10-µl pipette tip to remove cells by six perpendicular linear scratches. Repopulation of the wound surface was photographed near the crossing point of perpendicular scratches. Differences were calculated by comparing the zero time repopulation with 12 hours repopulation for each condition that was expressed as a percent wound closure of the control, which was taken as 100%. Two independent experiments with a minimum of three observations for each condition were analyzed.

Fabrication of HSE wound healing model

Skin equivalent cultures were prepared and wounded *in vitro* as described previously by Garlick and Taichman (1992). Skin equivalent cultures were wounded 7 days after keratinocytes were seeded onto the collagen matrix. One week before cultures were to be wounded, an additional collagen matrix was fabricated and used as the substrate onto which the wounded skin equivalents were transferred. To generate wounds, the skin equivalent culture was removed from the insert membrane using 1.5 cm punch, and a 1.2-cm incision that penetrated the epidermis and collagen matrix was created by scalpel and transferred onto the 7 day-old collagen matrix to create an elliptical wound that was 3 mm at its greatest width. Wounded cultures were maintained at the air–liquid interface for 24, 48, and 72 hours at 37 °C in 7.5% CO_2 to monitor reepithelialization.

Proliferation assay

Before harvesting, skin equivalents or collagen gels were labeled with a 6 hours pulse of BrdU (Sigma, St Louis, MO) at a final concentration of 10 µM. Tissues were harvested and frozen in embedding medium (Triangle Biomedical, Durham, NC) after being placed in 2 M sucrose overnight at 4 °C. Frozen tissues were serially sectioned at 8 µm and stained with mAbs against BrdU (Boehringer-Mannheim, Indianapolis, IN) and counterstained with DAPI (4,6-diamidino-2-phenylindole) (Vector, Burlingame, CA). Fluorescence was visualized using a Nikon eclipse 80i microscope and images were captured using Diagnostic Instruments SPOT RT camera. The number of BrdU-positive cells was determined and expressed as a percent of total cells in the basal layer. Two independent experiments with a minimum of three observations for each condition were analyzed. For routine light microscopy, skin equivalent tissues were fixed in 4% neutral-buffered formalin, embedded in paraffin, and serially sectioned at 8 µm. Sections were stained with hematoxylin and eosin.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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